

Web-Accessible Database of *hsp65* Sequences from *Mycobacterium* Reference Strains^{▼†}

Jianli Dai,^{1,4*} Yuansha Chen,^{1,2} and Michael Lauzardo^{3,4}

Emerging Pathogens Institute, University of Florida, Gainesville, Florida 32611¹; Department of Pathology, Immunology, and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida 32610²; Southeastern National Tuberculosis Center, Gainesville, Florida 32611³; and Department of Medicine, College of Medicine, University of Florida, Gainesville, Florida 32610⁴

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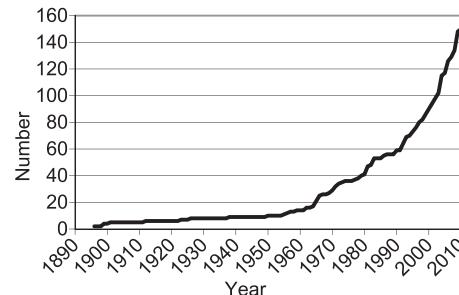
Mycobacteria include a large number of pathogens. Identification to species level is important for diagnoses and treatments. Here, we report the development of a Web-accessible database of the *hsp65* locus sequences (<http://msis.mycobacteria.info>) from 149 out of 150 *Mycobacterium* species/subspecies. This database can serve as a reference for identifying *Mycobacterium* species.

Included among the mycobacteria are a large number of clinically important pathogens, both obligate (e.g., *M. tuberculosis* and *M. leprae*) and opportunistic (e.g., *M. avium*, *M. kansasii*, etc.). The impact of mycobacteria on human morbidity and mortality is hard to overstate. Although tuberculosis (TB) is arguably one of the most important infectious diseases in the world, the incidence of disease due to nontuberculous mycobacteria (NTM) has been steadily increasing worldwide (6, 27, 42, 48, 59) and has likely far surpassed TB in the United States (7). Thus, the American Thoracic Society and the Infectious Diseases Society of America have recommended identifying the clinically significant NTM to the species level upon the diagnosis of nontuberculous mycobacterial diseases (17).

The *Mycobacterium* genus currently includes 150 species/subspecies (<http://www.bacterio.cict.fr/m/mycobacterium.html>), and the number has been increasing exponentially (Fig. 1), making identification difficult and challenging. Current identification based on biochemical tests of culture is slow and inadequate to differentiate among closely related mycobacteria, especially for those mycobacteria that are biochemically inert and slowly growing. In contrast, molecular identification methods based on PCR and nucleotide sequencing dramatically shorten the detection time and improve the accuracy of identification. The most common genomic loci used in molecular identification are the 16S rRNA gene (25), 16S-23S rRNA internal transcribed spacer (15), *hsp65* (40, 47), and *rpoB* (22). Almost all recent publications on new mycobacterial species compared sequences from multiple loci to those of established species, and *hsp65* is always included. A previous study has shown a 99.1% agreement between identification using *hsp65* sequencing and a conventional method combining Accuprobes, biochemical test panels, or 16S rRNA gene sequencing (33), suggesting that *hsp65* sequencing is an effective method for identifying mycobacterial species. It was also suggested that

completeness of the sequence database was critical for this identification method (33). To our knowledge, there is no publicly accessible *hsp65* sequence database that covers all currently validated mycobacterial species. Laboratorians and researchers have to rely on the sequences deposited in public databases, such as GenBank, EMBL, and DDBJ. The vast majority of these mycobacterial entries are from uncharacterized strains or undetermined species, and problematic sequence entries, such as base errors, incomplete sequences, invalid or misidentified species, and even species-strain mismatches, are frequently present. This makes identification by searching public databases onerous and error prone. To facilitate the taxonomic identification of mycobacterial isolates, we have developed a Web-accessible database of mycobacterial *hsp65* sequences from 149 species/subspecies, excluding the problematic GenBank entries mentioned above.

The type strain *hsp65* sequences of 147 mycobacterial species/subspecies were downloaded from GenBank. *M. lepraemurium* and *M. leprae* do not have type strains due to difficult cultivation. We chose *M. lepraemurium* TS130 and *M. leprae* TN as their reference strains and obtained their *hsp65* sequences with the GenBank sequence accession numbers AY550232 (34) and NC_002677 (11). Multiple identical GenBank sequences from the type strain of the same species were combined into a single entry in our database (Table 1). We trimmed the sequences to 401 bp, which corresponds to nucleotide positions 165 to 565 of the *M. tuberculosis* H37Rv *hsp65*



* Corresponding author. Mailing address: 2055 Mowry Rd., Rm. 275, Gainesville, FL 32611. Phone: (352) 273-9429. Fax: (352) 273-9430. E-mail: dai.jian.li@ufl.edu.

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FIG. 1. Numbers of approved *Mycobacterium* species/subspecies from 1890 to 2010.

TABLE 1. List of the *Mycobacterium* species/subspecies, the reference strains (all are type strains except *M. leprae* TN and *M. lepraemurium* TS130), and the GenBank sequence accession numbers for their *hsp65* sequences from published studies used to generate the database

Species/subspecies ^a	Reference strain	GenBank accession no. (reference[s]) ^b
<i>M. abscessus</i>	CIP 104536 ATCC 19977	AY458075 (2), AF547802 (19) EF486338 (24), AY498743 (43), NC_010397 (41), JF491290
<i>M. africanum</i>	CIP 105147 ATCC 25420	AF547803 (14) FJ617583 (20), JF491313
<i>M. agri</i>	CIP 105391	AY438080
<i>M. aichiense</i>	ATCC 27280 DSM 44147	AY299147 (23) AF547804 (14)
<i>M. alvei</i>	CIP 103464	AF547805 (14)
<i>M. aromaticivorans</i>	JS19b1	DQ841182 (19)
<i>M. arosiense</i>	DSM 45069 T1921	JF491321 EU370531 (4)
<i>M. arupense</i>	ATCC BAA-1401 DSM 44942 AR30097	GQ153297 (49) EU191917, GQ214503 (29), JF491325 DQ168662 (9)
<i>M. asiaticum</i>	ATCC 25276	AY299133 (23), GU362517 (13)
<i>M. aubagnense</i>	CIP 108543 CCUG 50186	AY859677 (1) DQ987727
<i>M. aurum</i>	ATCC 23366 CIP 104465	AF350414 (64), FJ172326 (44) AY438081
<i>M. austroafricanum</i>	CIP 105395	AF547807 (14)
<i>M. avium</i> subsp. <i>avium</i>	ATCC 25291 CIP 104244	AF126030 (28), EU239779 (5), GQ153289 (49), JF491291 AF547808 (14)
<i>M. avium</i> subsp. <i>paratuberculosis</i>	CIP 103963	AF547809 (14)
<i>M. avium</i> subsp. <i>silvicum</i>	ATCC 19698 ATCC 49884	AY299137 (23) EU239781 (5)
<i>M. boenickeyi</i>	CIP 103317	AF547810 (14)
<i>M. boemicum</i>	CIP 107829	AY943195
<i>M. bolletii</i>	CIP 105811 CCUG 50184	AF547811 (14) DQ987724
<i>M. botniense</i>	CIP 108541	EU266576, AY859675 (1), FJ607778 (26)
<i>M. bouchedurhonense</i>	DSM 44537	AF547812 (14)
<i>M. bovis</i>	CIP 109827	HM602039
<i>M. branderi</i>	CIP 105234	AF547813 (14)
<i>M. brisbanense</i>	ATCC 19210	JF491332
<i>M. brumae</i>	CIP 104592	AF547815 (14)
<i>M. canariense</i>	DSM 44680	AB456564, JF491333
<i>M. caprae</i>	CIP 107830	AY943196
<i>M. celatum</i>	CIP 103465	AF547816 (14)
<i>M. chelonae</i>	502329 DSM 44828	AY255477 (21) JF491316
<i>M. chimaera</i>	CIP 105776	AF547884 (14)
<i>M. chitae</i>	ATCC 51131	AY299180 (23), JF491292
<i>M. chlorophenolicum</i>	CIP 106109	AF547817 (14)
<i>M. chubuense</i>	CIP 104535	AF547818 (14), AY458074 (2)
<i>M. colombense</i>	ATCC 35752	JF491293
<i>M. conceptionense</i>	CIP 104189	GQ153296 (49), AY943198
<i>M. confluens</i>	CIP 106810	EU239783 (5)
<i>M. conspicuum</i>	CIP 108962	AF547819 (14)
<i>M. cookie</i>	CIP 108544	AY299149 (23)
<i>M. cosmeticum</i>	CIP 105510	AF547820 (14)
<i>M. crocinum</i>	CIP 105165	AF547821 (14)
<i>M. diernhoferi</i>	CIP 105396	EU239785 (5), GQ153298 (49)
<i>M. doricum</i>	LTA-388	AM902957 (39), EU191920, AY859678 (1)
<i>M. duvalii</i>	DSM 44829	AF547822 (14)
<i>M. elephantis</i>	czh-42	AF547823 (14)
<i>M. fallax</i>	CIP 105384	AF547824 (14)
	DSM 44339	AY449730 (12)
	CIP 104539	DQ124111
	CIP 106831	DQ533998 (19)
	CIP 81.39	AF547825 (14)
	ATCC 35219	AF547826 (14)
		AF547827 (14)
		AF547828 (14)
		AF547829 (14)
		JF491294

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TABLE 1—Continued

Species/subspecies ^a	Reference strain	GenBank accession no. (reference[s]) ^b
<i>M. farcinogenes</i>	ATCC 35753 DSM 43637 NCTC 10955	AY299150 (23) AF547830 (14) AY458073 (2)
<i>M. flavescens</i>	ATCC 14474	AF350413 (64), GU362519 (13)
<i>M. florentinum</i>	DSM 44852	DQ350162, JF491317
<i>M. fluoranthenivorans</i>	DSM 44556	DQ350157, JF491318
<i>M. fortuitum</i> subsp. <i>acetamidolyticum</i>	CIP 105423 ATCC 35931	AF547832 (14) JF491314
<i>M. fortuitum</i> subsp. <i>fortuitum</i>	CIP 104534 ATCC 6841	AF547833 (14) JF491295
<i>M. frederiksbergense</i>	DSM 44346	AF547834 (14)
<i>M. gadium</i>	CIP 105388	AF547835 (14)
<i>M. gastri</i>	CIP 104530 ATCC 15754	AF547836 (14) JF491315
<i>M. genavense</i>	DSM 44424	AF547837 (14)
<i>M. gilvum</i>	DSM 44503	AF547838 (14)
<i>M. goodii</i>	CIP 106349	AF547839 (14)
<i>M. gordonaiae</i>	ATCC 700504 CIP 104529	AY458071 (2) AF547840 (14)
<i>M. haemophilum</i>	ATCC 14470 CIP 105049	AF434734 AF547841 (14)
<i>M. hassiacum</i>	ATCC 29548	AY299185, GQ245967, JF491296
<i>M. heckeshornense</i>	CIP 105218	AF547842 (14)
<i>M. heidelbergense</i>	DSM 44428	AF547843 (14)
<i>M. hiberniae</i>	CIP 105424	AF547844 (14)
<i>M. hiberniae</i>	DSM 44241	AY438083
<i>M. hodleri</i>	ATCC 49874	JF491297
<i>M. holsaticum</i>	CIP 104909	AF547845 (14)
<i>M. houstonense</i>	DSM 44478	AY438084
<i>M. immunogenum</i>	ATCC 49403	AY458077 (2)
<i>M. insubricum</i>	DSM 44676	DQ987725
<i>M. interjectum</i>	CIP 106684	AY458081 (2), EU266577
<i>M. intermedium</i>	DSM 45132	JF491319
<i>M. intracellularare</i>	FI-06250	EF584487 (51)
<i>M. kansasii</i>	DSM 44064	AF547846 (14)
<i>M. komossense</i>	ATCC 51457	JF491298
<i>M. kubicae</i>	CIP 104542	AF547847 (14)
<i>M. kumamotoense</i>	ATCC 51848	AY299187
<i>M. kyorinense</i>	ATCC 13950	AF126035 (28), DQ284774 (53), GQ153290 (49), JF491299
<i>M. lacus</i>	TMC 1406	U85633 (46)
<i>M. leprae</i>	CIP 104589	AF547849 (14)
<i>M. lepraeumurium</i>	ATCC 12478	AF434739, AY299189, JF491300
<i>M. lentiflavum</i>	CIP 105293	AY438649
<i>M. llatzerense</i>	CIP 106428	AF547850 (14)
<i>M. madagascariense</i>	ATCC 700732	AY373458 (23)
<i>M. mageritense</i>	CST 7247	AB239920 (32)
<i>M. malmoense</i>	CCUG 51961	EU191915
<i>M. mantenii</i>	DSM 45093	JF491323
<i>M. marinum</i>	KUM 060204	AB370171 (37)
<i>M. marseillense</i>	DSM 45166	HM602040
	DSM 44577	AY438090
	TN	NC_002677 (11)
	TS130	AY550232 (34)
	CIP 105465	AF547851 (14)
	MG13	AM421341 (16)
	DSM 45343	JF491330
	CIP 104538	AF547852 (14)
	CIP 104973	AY458070 (2), AF547853 (14)
	CIP 105775	AF547854 (14)
	ATCC 29571	GQ153293 (49), JF491301
	NLA000401474	FJ232523 (60)
	CIP 109863	HM602041
	ATCC 927	AY299134 (23), AF456470 (55), AB548715
	NCTC 2275	AF271346 (45)
	CIP 104528	AF547855 (14)
	5356591	EU239787 (5)
	CIP 109828	HM602037

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TABLE 1—Continued

Species/subspecies ^a	Reference strain	GenBank accession no. (reference[s]) ^b
<i>M. massiliense</i>	CIP 108297 CCUG 48898	EU191919, EF486339 (24), EU266578 AY596465 (3)
<i>M. microti</i>	CIP 104256 ATCC 19422	AF547856 (14) AY299135 (23)
<i>M. monacense</i>	DSM 44395	EU191918, JF491320
<i>M. montefiorens</i>	DSM 44602 ATCC BAA-256	AY943204 AY027785 (31)
<i>M. moriokaense</i>	CIP 105393	AF547857 (14), AY859680 (1)
<i>M. mucogenicum</i>	ATCC 49650	AY299155, AY458079 (2)
<i>M. murale</i>	CIP 105980	AF547859 (14)
<i>M. nebraskense</i>	DSM 44803 ATCC BAA-837	DQ124110 GQ153294 (49)
	UNMC-MY1349	AY368457 (35)
<i>M. neoaurum</i>	ATCC 25795	AY299156, FJ172320 (44), JF491302
	CIP 105387	AF547860 (14)
<i>M. neworleansense</i>	CIP 107827	AY943199
	ATCC 49404	AY458076 (2), AY496143 (62)
<i>M. nonchromogenicum</i>	DSM 44164 ATCC 19530	AF547861 (14) AY299136 (23), AF434732, JF491303
<i>M. noviomagense</i>	NLA000500338	EU600390 (57)
<i>M. novocastrense</i>	CIP 105546	AF547862 (14)
<i>M. obuense</i>	CIP 106803	AF547863 (14)
<i>M. pallens</i>	czh-8	DQ533997 (19)
<i>M. palustre</i>	DSM 44572	AY943200
<i>M. paraffinicum</i>	ATCC 12670	GQ153287 (49)
<i>M. parafortuitum</i>	CIP 106802	AF547864 (14)
<i>M. parascrofulaceum</i>	ATCC BAA-614 CIP 108112	AY337274 (52), GQ153295 (49) AY943201
<i>M. paraseoulense</i>	DSM 45000 31118	HM602042, JF491324 DQ536402
<i>M. parmense</i>	CIP 107385	HM022199
<i>M. peregrinum</i>	NCTC 10264	AM902953 (39)
	CIP 105382	AY458069 (2), AF547865 (14)
	ATCC 14467	AY299159 (23)
<i>M. phlei</i>	ATCC 11758	AY299158 (23)
	CIP 105389	AF547866 (14)
<i>M. phocaicum</i>	CCUG 50185	DQ987726
	CIP 108542	AY859676 (1), EU266579
<i>M. porcinum</i>	ATCC 33776	AY496137 (62), JF491326
<i>M. poriferae</i>	CIP 105394	AF547868 (14)
<i>M. pseudoshottsii</i>	NCTC 13318 ATCC BAA-883	AM902956 (39), DQ987722 AY571788
<i>M. psychrotolerans</i>	DSM 44697	HM602035
<i>M. pulveris</i>	CIP 106804	AF547869 (14)
<i>M. pyrenivorans</i>	DSM 44605	JF510463
<i>M. rhodesiae</i>	CIP 106806	AF547870 (14)
<i>M. riyadhense</i>	NLA000201958	EU921671 (56)
<i>M. rufum</i>	JS14	DQ841181 (19)
<i>M. rutilum</i>	czh-117	DQ841180 (19)
<i>M. salmoniphilum</i>	ATCC 13758	DQ866777 (63)
<i>M. saskatchewanense</i>	NRCM 00-250; ATCC BAA-544 CIP 108114	AY208858 (54) AY943203
	DSM 44616	JF491331
<i>M. scrofulaceum</i>	ATCC 19981	GQ153288 (49), AF434733, AY299138 (23), JF491304
	CIP 105416	AF547871 (14)
<i>M. senegalense</i>	NCTC 10956	AM902954 (39)
	ATCC 35796	AY684045 (61), JF491327
<i>M. senuense</i>	DSM 44999	FJ268582, JF491328
	05-832	DQ536409 (36)
<i>M. seoulense</i>	DSM 44998	EU191916, JF491322
<i>M. septicum</i>	ATCC 700731	AY373457 (23), AY496142 (62)
	DSM 44393	JF491329
<i>M. setense</i>	CIP 109395	EU371505 (50)
<i>M. shimoidei</i>	DSM 44152 ATCC 27962	AF547874 (14)
	NCTC 13215T	JF491305
<i>M. shottsi</i>	ATCC 700981	AM902955 (39), DQ987723 AY550225 (34), EU619895

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TABLE 1—Continued

Species/subspecies ^a	Reference strain	GenBank accession no. (reference[s]) ^b
<i>M. simiae</i>	CIP 104531	AF547875 (14)
	ATCC 25275	GQ153292 (49), AF434730, JF491306
<i>M. smegmatis</i>	ATCC 19420	AY458065 (2), JF491307
	CIP 104444	AF547876 (14)
<i>M. sphagni</i>	DSM 44076	AF547877 (14)
<i>M. stomatepiae</i>	DSM 45059T	AM902968 (38, 39)
<i>M. szulgai</i>	ATCC 35799	AF350412 (64), AY299141 (23), JF491308
	CIP 104532	AF547878 (14)
<i>M. terrae</i>	ATCC 15755	AF257468, AF434736, AY299142 (23)
	CIP 104321	AF547879 (14)
<i>M. thermoresistibile</i>	CIP 105390	AF547880 (14)
<i>M. timonense</i>	CIP 109830	HM602038
<i>M. tokaiense</i>	CIP 106807	AF547881 (14)
	ATCC 27282	JF491309
<i>M. triplex</i>	ATCC 700071	AY027786 (31), GQ153291 (49)
	CIP 106108	AF547882 (14)
<i>M. triviale</i>	DSM 44153	AF547883 (14)
	ATCC 23292	AF434737, AY299143 (23), JF491310
<i>M. tuberculosis</i>	ATCC 27294	AY299144 (23), JF491311
	H37Rv	NC_000962 (8, 10)
<i>M. tusciae</i>	CIP 106367	AF547887 (14)
<i>M. ulcerans</i>	ATCC 19423	AY299145 (23), AB548723, AF271096 (45)
<i>M. vaccae</i>	CIP 105934	AF547889 (14)
	ATCC 15483	JF491312
<i>M. vanbaalenii</i>	DSM 7251	AY438091
	PYR-1	NC_008726
<i>M. vulgaris</i>	NLA000700772	EU834054 (58)
<i>M. wolinskii</i>	CIP 106348	AF547890 (14)
	ATCC 700010	AY299164 (23), AY458064 (2)
<i>M. xenopi</i>	CIP 104035	AF547891 (14)
	ATCC 19250	AF434738, AY373454 (23)

^a The sequences of 47 species/subspecies (boldface) were validated by our laboratory. The sequences of 40 species/subspecies (underlined) are supported by multiple GenBank records deposited by other research groups.

^b Accession numbers in bold are for sequences determined in our laboratory.

gene and can be amplified and sequenced using primers Tb11 and Tb12 (47). Entries that did not completely cover this 401-bp *hsp65* locus were excluded from our database. Using previously described methods (13), we determined and verified 47 *hsp65* sequences and submitted them to GenBank (bold accession numbers in Table 1). As a result of our verification, the incorrect sequences from *M. asiaticum*, *M. flavescent*, *M. intracellulare*, *M. porcinum*, *M. senegalense*, *M. septicum*, and *M. szulgai* in GenBank were excluded from our database, and only verified sequences of these species were adopted. Sequence similarities were determined by MEGA 5.02 (<http://www.megasoftware.net/>). Currently, there are a total of 143 unique sequences from 149 species/subspecies in this database. Identical sequences are found among the three *M. avium* subspecies, between two *M. fortuitum* subspecies, and among four *M. tuberculosis* complex members (i.e., *M. bovis*, *M. caprae*, *M. microti*, and *M. tuberculosis*). A BLAST server based on this database has been developed (<http://msis.mycobacteria.info>). Query sequences are accepted in FASTA format by copying and pasting or file uploading and then searched against the database using NCBI's BLASTN program. The output results will show 20 best hits to suggest their taxonomic categories at species level. The pairwise alignments and the percentages of identities are shown in the results as well. PhyML 3.0 (18) was used to generate a maximum-likelihood phylogeny of these 149 *Mycobacterium* species/subspecies (Fig. 2) that is the most complete so far, covering 99.3% of the *Mycobacterium* genus

(the only missing species is *M. pinnipedii*, an *M. tuberculosis* complex member). Slowly growing mycobacteria (SGM) and rapidly growing mycobacteria (RGM) are clearly separated, except that the slowly growing *M. tusciae*, *M. hiberniae*, *M. nonchromogenicum*, and *M. triviale* are grouped with the RGM.

The popularity of species identification using *hsp65* sequences has resulted in a large number of mycobacterial *hsp65* sequences being deposited in public repositories. McNabb et al. also developed an in-house database, including 111 *Mycobacterium* species (34). The accuracy and coverage are crucial for the database to become a viable solution for species identification. Here, we report a publicly accessible *hsp65* database with 99.3% coverage of the entire *Mycobacterium* genus, of which 47 species/subspecies have been verified in our laboratory (boldface in Table 1) and 40 entries are supported by multiple GenBank sequences and thus are considered confirmed sequences (underlined in Table 1). A 97% identity was previously suggested as a criterion for identifying a species using *hsp65* sequences (34). However, pairwise comparison of these 149 species identified 219, 121, and 45 instances of sequence similarity greater than 97%, 98%, and 99%, involving 94 (63.1%), 82 (55.0%), and 42 (28.2%) species/subspecies, respectively (see the table in the supplemental material). This makes the identification of species with less than 100% matches challenging. Because the interspecies similarities vary from group to group in the phylogeny and change as the number of species/subspecies increases, investigators need to be

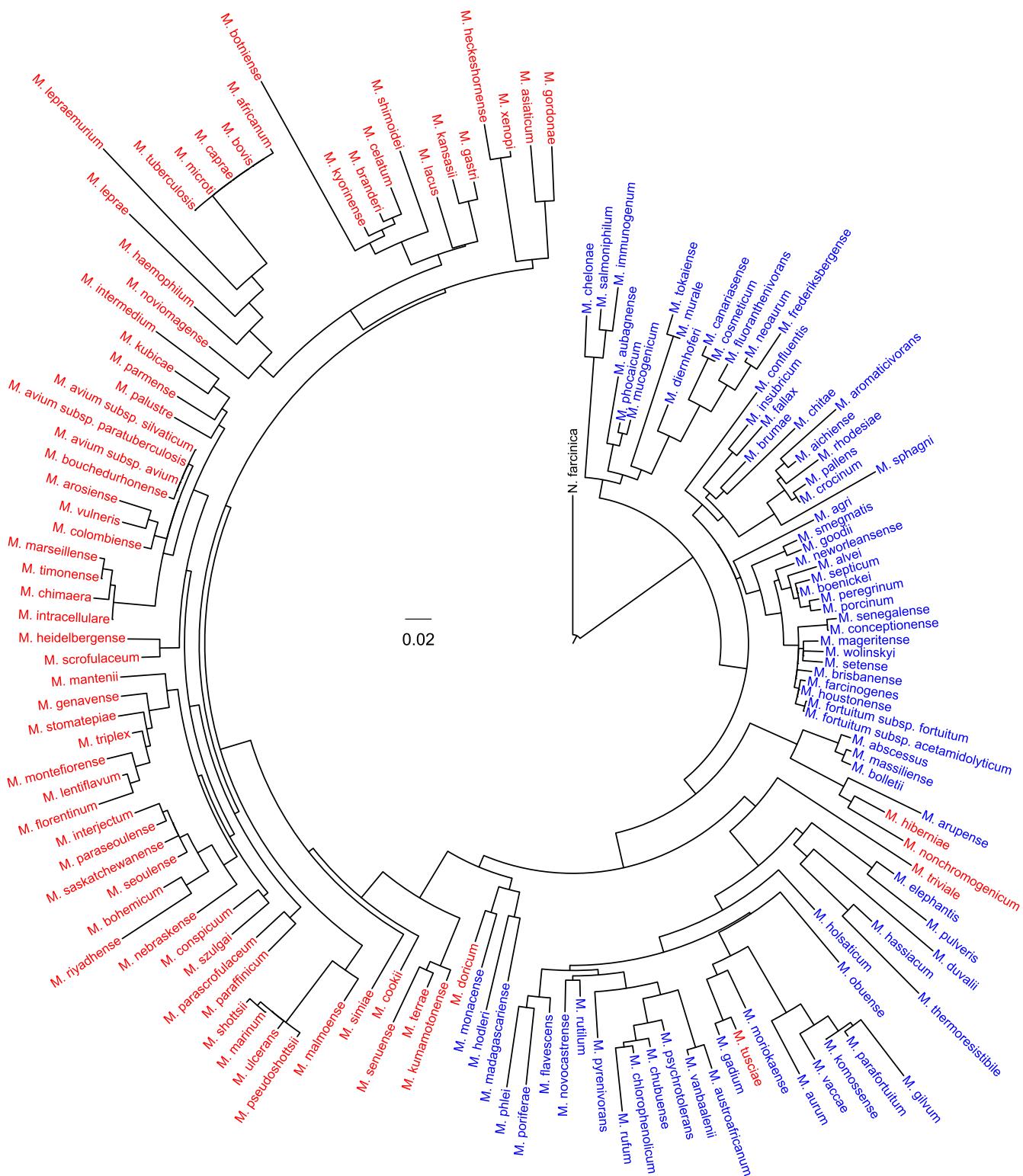


FIG. 2. Maximum-likelihood phylogeny of *Mycobacterium* genus. PhyML 3.0 with default settings (18) and iTOL (30) were used to generate the circular phylogenetic tree rooted with *Nocardia farcinica* (strain IFM 10152). Rapidly growing mycobacteria are in blue, while slowly growing mycobacteria are in red. The scale bar is equivalent to 0.02 substitutions/site.

cautious when assigning species/subspecies for these isolates. Further research is needed to establish a more reliable criterion and validate our database with clinical and environmental isolates. Nevertheless, our database provides the most comprehensive phylogenetic information on the mycobacterial *hsp65* locus that can facilitate species identification in this genus.

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